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Electrochemical, Antifungal, Antibacterial and DNA Cleavage Studies of Some Co(II), Ni(II), Cu(II) and Zn(II)-Copolymer Complexes

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Cyclic voltammetric measurements were performed for Co(II), Ni(II), Cu(II) and Zn(II) complexes of 1 : 1 alternating copolymer, poly(3-nitrobenzylidene-1-naphthylamine-co-succinic anhydride) (L) and Ni(II) and Cu(II) complexes of 1 : 1 alternating copolymer, poly(3-nitrobenzylidene-1-naphthylamine-co-methacrylic acid) (L^1). The *in vitro* biological screening effects of the investigated compounds were tested against the fungal species including *Aspergillus niger*, *Rhizopus stolonifer*, *Aspergillus flavus*, *Rhizoctonia bataicola* and *Candida albicans* and bacterial species including *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumaniae*, *Proteus vulgaris* and *Pseudomonas aeruginosa* by well diffusion method. A comparative study of inhibition values of the copolymers and their complexes indicates that the complexes exhibit higher antimicrobial activity. Copper ions are proven to be essential for the growth-inhibitor effect. The extent of inhibition appeared to be strongly dependent on the initial cell density and on the growth medium. The nuclease activity of the above metal complexes were assessed by gel electrophoresis assay and the results show that the copper complexes can cleave pUC18 DNA effectively in presence of hydrogen peroxide compared to other metal complexes. The degradation experiments using Rhodamine B dye indicate that the hydroxyl radical species are involved in the DNA cleavage reactions.

KEYWORDS : Copolymer, Growth-inhibitor, Inhibition, DNA

Designing of metal complexes for cleaving DNA is currently an area of considerable interest from chemical as well as biological stand points and offers potential applications in the field of medicine in the post-genomic era. Transition metal complexes as artificial nucleases are a matter of an extensive research due to their diverse structural features and reactivities (Pretviel *et al*., 1995). Exploration of Cu(II) complexes as chemical nucleases has seen an upsurge in recent years due to biologically accessible redox potential and high affinity toward nucleobases. There are several reports in the literature (De Silveira *et al*., 2008) on copper-induced DNA cleavage using various exogenously added co-reactants such as hydrogen peroxide, peroxyacids and thiols. However, very little attention is paid on coordinated polynuclear complexes (Srivatsan *et al*., 2003). With their versatile structures, redox behaviour and physicochemical properties, these complexes are often useful as chemical nucleases (Macías *et al*., 2007; Liu *et al*., 2004). It has been reported that many transition metal complexes have activity of chemistry nuclease, such as splitting DNA with specificity. The synthesis of polymers containing reactive functional groups has been an active field of research in polymer science, because it provides an approach to a subsequent modification of the polymer for the required application. Polymer bound chelating ligands including polydentate amines, Schiff bases and porphyrins have been described (Drago *et al*., 1980). In our laboratory, some copolymers and their metal(II) complexes have been synthesized and characterized (unpublished data) to explore the catalytic potential of nucleic acid cleavage. The present paper deals with the electrochemical, antifungal, antibacterial and DNA cleavage studies of Co(II), Ni(II), Cu(II) and Zn(II) complexes of 1 : 1 alternating copolymer, poly(3-nitrobenzylidene-1-naphthylamine-co-succinic anhydride) (L) and Ni(II) and Cu(II) complexes of 1 : 1 novel alternating copolymer, poly(3-nitrobenzylidene-1-naphthylamine-co-methacrylic acid) (L^1). The preparation of these complexes and their characterization by elemental analysis, molar conductance, IR, UV-Vis., NMR, Magnetic measurements, ESR, Thermal Studies, XRD and SEM, have already been reported.

Materials and Methods

Apparatus and reagents. Cyclic Voltammetric studies were performed at a scan rate of 0.1 Vs^{-1} using BAS CV-50W electrochemical analyzer/EG & G Princeton Applied Research Potentiostat/Galvanostat Model 273A, controlled by M270 software and by using a glassy carbon working electrode, Pt wire auxillary electrode and an Ag/AgCl reference electrode. $LiClO_4$ was used as the supporting electrolyte. Supercoiled plasmid DNA (pUC18), agarose and ethidium bromide used are of extra pure samples. All buffer solutions were prepared using deionised, double distilled water as described elsewhere (Bidyut *et al*.,

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2002).

Antimicrobial activity. The *in vitro* evaluation of antimicrobial activity was carried out. The purpose of the screening program is to provide antimicrobial efficiencies of the investigated compounds. The prepared compounds were tested against some fungal and bacterial species to provide the minimum inhibitory concentration (MIC) for each compound. MIC is the lowest concentration of solution to inhibit the growth of a test organism.

***In vitro* antimicrobial activity.** Antimicrobial activity of the copolymers and their complexes were tested against the fungal species including *Aspergillus niger*, *Rhizopus stolonifer*, *Aspergillus flavus*, *Rhizoctonia bataicola* and *Candida albicans*, cultured on potato dextrose agar medium and also performed by the well diffusion method. The ligands and their complexes were tested against the bacterial species including *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumaniae*, *Proteus vulgaris* and *Pseudomonas aeruginosa*, cultured on nutrient agar medium by the well diffusion method. Streptomycin was used as standard antibacterial agent. The compounds were prepared in DMSO. In a typical procedure, a well was made on the agar medium inoculated with microorganisms. The well was filled with the test solution using a micropipette and the plates were incubated at 37°C for 72 h for fungi and 24 h for bacteria. During this period, the test solution diffused and affected the growth of the inoculated fungi and bacteria. The zone of inhibition, developed on the plate was measured. The MIC of all synthesized compounds was determined by the method of serial dilution technique.

Verification of DNA purity. Solutions of CT DNA in 5 mM Tris-HCl/50 mM NaCl (pH = 7.1) gave a ratio of UV absorbance at 260 and 280 nm, A_{260}/A_{280} of *ca.* 1.8~1.9, indicating that DNA was sufficiently free of protein contamination. The DNA concentration was determined by the UV absorbance at 260 nm after 1 : 100 dilutions. The molar absorption coefficient was taken as 6600 M^{-1}. Stock solutions were kept at 4°C and used within 4 days.

Gel electrophoresis. Cleavage reactions were run between the metal-polymer complexes and DNA, and the solutions were diluted with loading dye using 1% agarose gel. 1 × Tris acetate EDTA buffer was prepared by mixing an appropriate concentration of 1 *ml* of 50X TAE buffer with 49 mL distilled water, followed by the addition of 0.5 g of powdered agarose and mixed well. The solution was heated to boiling to dissolve agarose completely. The completely dissolved agarose gel solution was kept in the water bath at 65°C. Then 3 μl of Ethidium bromide (0.5 $\mu g/ml$) was added to the above solution and mixed well. The warmed agarose was poured and clamped immediately with comb to form sample wells. After setting (30~45 min at room temperature) the comb was removed and taped. The gel was mounted into electrophoretic tank where the distance between the electrodes was 19 cm. Enough electrophoretic buffers were added to cover the gel to a depth of about 1 mM. The DNA sample (30 μM), 50 μM metal complex, 500 μM H_2O_2 in 50 mM Tris-HCl buffer (pH = 7.1) were mixed with loading dye and loaded into the well of the submerged gel using a micropipette. The electric current was passed into running buffer. The sample was running from negative pole to positive pole. After 1~2 h the gel was taken out from the buffer. After electrophoresis, the gel was photographed under UV transluminator and documented.

Results and Discussion

Electrochemical studies. The cyclic voltammetric data of the complexes are presented in Table 1. The cyclic voltammogram of the $[CoL(OAc)_2]$ complex in acetonitrile solution at 300°K in the potential range −1.3 to +1.7 V was recorded. It shows two irreversible peaks observed at −0.848 and 1.012 V. It corresponds to the Co(II)/Co(III) and ligand oxidation. The cyclic voltammogram of the $[NiL(OAc)_2]$ complex in acetonitrile solution at 300°K in the potential range −1.3 to +1.7 V was recorded. It shows well defined redox process corresponding to the formation of the quasi-reversible Ni(II)/Ni(III) couple. The anodic peak at Epa = −0.187 V *versus* Ag/AgCl and the associated cathodic peak at Epc = −0.881 V correspond to Ni(II)/Ni(III) couple. It also shows an irreversible peak observed at Epa = 1.076 V corresponds to Ni(II)/Ni(I). The $[NiL(OAc)_2]$ complex exhibits a quasi-reversible behaviour as indicated by the non-equivalent current intensity of cathodic and anodic peaks. The high negative potential shows that the metal ion is highly stabilized by the strong π-donor properties of the substituents which overcomes

Table 1. Cyclic voltammetric data of metal(II) copolymer complexes

Complex	Couple/ Peak	Cathodic, Epc (V)	Anodic, Epa (V)	ΔEp (V)
$[CoL](OAc)_2$	II/III	−0.848	–	–
		–	1.012	–
$[NiL](OAc)_2$	II/III	−0.881	−0.187	0.694
	II/I Peak	–	1.076	–
$[CuL](OAc)_2$	II/I	−0.948	−0.548	0.400
$[ZnL](OAc)_2$	II/0 Peak	−0.813	–	–
$[NiL^1(OAc)_2]$	II/I Peak	–	1.044	–
	II/III Peak	−0.799	–	–
$[CuL^1(OAc)_2]$	II/I	−0.903	−0.526	0.378

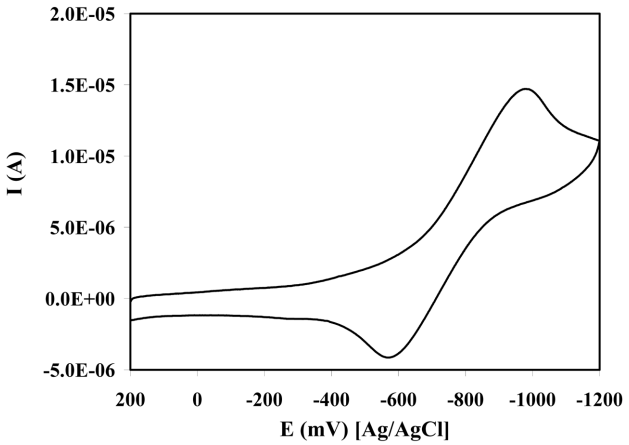

Fig. 1. Cyclic voltammetric profile of $[CuL](OAc)_2$ complex.

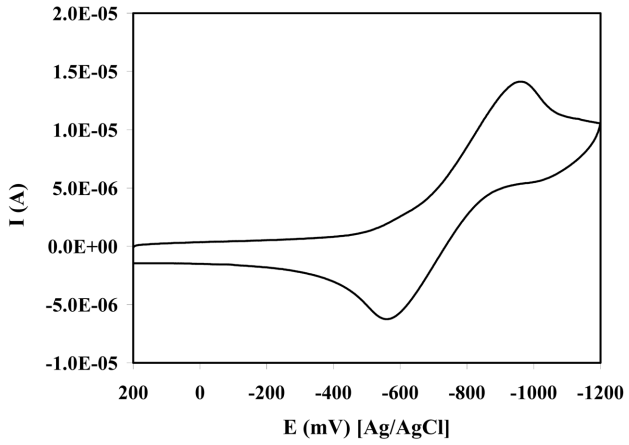

Fig. 2. Cyclic voltammetric profile of $[CuL^1](OAc)_2$ complex.

the π-acceptor properties of the ligand L. The cyclic voltammogram of the $[CuL(OAc)_2]$ complex in acetonitrile solution at 300°K in the potential range +0.2 to −1.2 V at scan rate 0.1 Vs^{-1} was recorded (Fig. 1). It shows a well defined redox process corresponding to the formation of the quasi-reversible Cu(II)/Cu(I) couple. The anodic peak at Epa = −0.548 V *versus* Ag/AgCl and the associated cathodic peak at Epc = −0.948 V correspond to the Cu(II)/Cu(I) couple. The cyclic voltammogram of the $[ZnL(OAc)_2]$ complex in acetonitrile solution at 300°K in the potential range −1.3 to +1.7 V shows an irreversible peak at −0.813 V, corresponding to Zn(II)/Zn(0)couple.

The cyclic voltammogram of the $[NiL^1(OAc)_2]$ complex in acetonitrile solution at 300°K in the potential range −1.3 to +1.7 V was recorded. It shows two irreversible peaks observed at −0.799 and 1.044 V. It corresponds to the formation of Ni(II)/Ni(I) and Ni(II)/Ni(III). The cyclic voltammogram of the $[CuL^1(OAc)_2]$ complex in acetonitrile solution at 300°K in the potential range +0.2 to −1.2 V was recorded (Fig. 2). It shows a well defined redox process corresponding to the formation of the quasi-reversible Cu(II)/Cu(I) couple. The anodic peak at Epa = −0.526 V *versus* Ag/AgCl and the associated cathodic peak at Epc = −0.903 V correspond to the Cu(II)/Cu(I) couple. The $[CuL^1(OAc)_2]$ complex exhibits quasi-reversible behaviour as indicated by the non-equivalent current intensity of cathodic and anodic peaks. The quasi reversible behaviour of this complex is also supported by the presence of large peak separation of E_{pa} and E_{pc}.

Antimicrobial activity. The *in vitro* antimicrobial activity of the compounds was tested against the fungal species including *Aspergillus niger*, *Rhizopus stolonifer*, *Aspergillus flavus*, *Rhizoctonia bataicola* and *Candida albicans* and the bacterial species including *Escherichia coli*, *Salmonella typhi*, *Staphylococcous aureus*, *Klebsiella pneumoniae* and *Pseudomonous aeruginosa* by well diffusion method. The MIC values of the compounds are summarized in Tables 2 and 3. A comparative study of the copolymers and their metal complexes (MIC values) indicates that complexes exhibit higher antifungal and antibacterial activity compared to those of the free copolymers. Compounds containing >C=N group have enhanced

Table 2. Minimum inhibitory concentration of the synthesised compounds against the growth of fungi ($\mu g/ml$)

Compound	*A. niger*	*R. stolonifer*	*A. flavus*	*R. bataicola*	*C. albicans*
L	76	84	104	92	98
L^1	62	68	82	85	68
$[CoL](OAc)_2$	52	56	42	76	60
$[NiL](OAc)_2$	46	52	38	72	52
$[CuL](OAc)_2$	38	46	30	64	40
$[ZnL](OAc)_2$	58	70	66	84	68
$[NiL^1(OAc)_2]$	56	48	32	36	30
$[CuL^1(OAc)_2]$	42	52	60	46	72
Nystatin[a]	10	16	8	12	14

[a]Standard

L = poly(3-nitrobenzylidene-1-naphthylamine-co-succinic anhydride

$\mathbf{L^1}$ = poly(3-nitrobenzylidene-1-naphthylamine-co-methacrylicacid)

Table 3. Minimum inhibitory concentration of the synthesized compounds against the growth of bacteria ($\mu g/ml$)

Compound	*E. coli*	*K pneumonia*	*S. typhi*	*P. aeruginosa*	*S. aureus*
L	74	80	76	98	120
L^1	88	76	64	96	104
$[CoL](OAc)_2$	60	44	32	65	52
$[NiL](OAc)_2$	52	42	64	72	60
$[CuL](OAc)_2$	46	38	56	60	84
$[ZnL](OAc)_2$	64	38	46	72	48
$[NiL^1(OAc)_2]$	66	76	52	46	78
$[CuL^1(OAc)_2]$	52	46	60	76	80
Streptomycin[a]	04	08	10	06	12

[a]Standard
L = poly(3-nitrobenzylidene-1-naphthylamine-co-succinic anhydride)
$\mathbf{L^1}$ = poly(3-nitrobenzylidene-1-naphthylamine-co-methacrylic acid)

antimicrobial activity than >C=C< group. The growth of certain microorganisms takes place even in the absence of O_2. Hence, compounds containing >C=C< group though capable of absorbing O_2 are not related with the growth of microorganisms. Such increased activity of the complexes can be explained on the basis of Overtone's concept (Anjaneyulu *et al*., 1986) and Tweedy's Chelation theory (Prabhakaran *et al*., 2004). According to Overtone's concept of cell permeability, the lipid membrane that surrounds the cell favours the passage of only the lipid-soluble materials due to which liposolubility is an important factor, which controls the antifungal and antibacterial activity. Chelation reduces the polarity of the metal ion because of the partial sharing of its positive charge with the donor groups and possible π-electron delocalization over the chelate ring. Such chelation could increase the lipophilic character of the central metal atom. The increased lipophilicity subsequently favours the permeation through the lipid layer of cell membrane (Prabhakaran *et al*., 2004). These complexes also disturb the respiration process of the cell and thus block the synthesis of the proteins that restricts further growth of the organism. The variation in the effectiveness of different complexes against different organisms depend either on differences in the permeability of the cells of the microbes or on difference in ribosome of the microbial cells (Akmal *et al*., 2007).

Mode of action. Although the exact mechanism is not understood biochemically, mode of action of antimicrobials may involve various targets in microorganisms.

(i) Interference with the cell wall synthesis, damage as a result of which cell permeability may be altered (or) they may disorganize the lipoprotein leading to the cell death.

(ii) Deactivate various cellular enzymes, which play a vital role in different metabolic pathways of these microorganisms.

(iii) Denaturation of one or more proteins of the cell, as a result of which the normal cellular processes are impaired.

(iv) Formation of a hydrogen bond through the azomethine group with the active centre of cell constituents, resulting in interference with the normal cell process (Akmal *et al*., 2007).

Effect of hetero atoms. From the observations (Tables 2 & 3), the higher inhibition of microbial growth is due to uncoordinated hetero atoms. In the complexes, the copolymers (L & L^1) have some uncoordinated donor atoms which enhance the activity of the complexes by bonding with the trace elements present in microorganisms. This

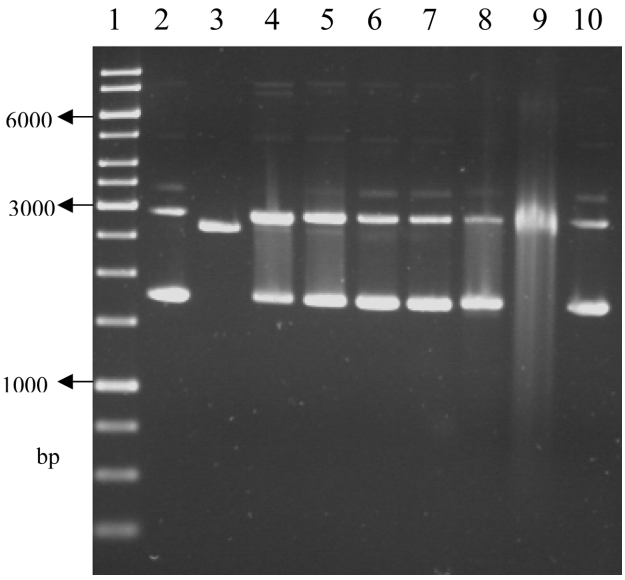

Fig. 3. Agarose gel (1%) showing the results of electrophoresis of pUC18 DNA with metal(II) polymer complexes. Lane 1:1K b ladder; Lane 2:pUC18; Lane 3:pUC18/*Hind*III; Lane 4:C1-$[CuL](OAc)_2$; Lane 5:C2-$[NiL](OAc)_2$; Lane 6:C3-$[CoL](OAc)_2$; Lane 7:C4-$[ZnL](OAc)_2$; Lane 8:C5-$[CuL^1](OAc)_2$; Lane 9:C6-$[NiL^1](OAc)_2$; Lane 10:Internal control.

can combine with the uncoordinated site and inhibit the growth of microorganisms.

Cleavage of pUC18 DNA. Gel electrophoresis experiments using pUC18 DNA were performed with metal-polymer complexes in the presence and absence of H_2O_2 as an oxidant. The nuclease activity is greatly enhanced by the incorporation of metal ion in the respective copolymer, it is evident from Fig. 3 that the complexes cleave DNA more efficiently in the presence of oxidant, which may be due to the formation of hydroxyl free radicals. The production of hydroxyl free radical is due to the reaction between the metal complex and oxidant (Surendra Babu *et al.*, 2007). These hydroxyl radicals participate in the oxidation of the deoxyribose moiety, followed by hydrolytic cleavage of the sugar phosphate backbone (Surendra Babu *et al.*, 2007). The more pronounced nuclease activity in the metal complexes in the presence of oxidant may be due to the increased production of hydroxyl radicals. The cleavage efficiency was measured by determining the ability of the complex to convert the Supercoiled DNA to nicked (open circular) form or sheared form. As is evident from Fig. 3, there is a considerable increase in the intensity of bands for open circular form in the case of samples $[CuL](OAc)_2$, $[CuL^1](OAc)_2$ and $[NiL](OAc)_2$. This suggests that samples $[CuL](OAc)_2$, $[CuL^1](OAc)_2$ and $[NiL](OAc)_2$ have nicking activity. However, there is no appreciable level of increase in the intensity of bands of open circular form for the samples treated with $[CoL](OAc)_2$ and $[ZnL](OAc)_2$ Shearing of plasmid DNA is evident in lane 9 where the plasmid DNA was treated with sample $[NiL^1](OAc)_2$. Further standardization of cleavage activity in terms of concentration of metal complex(es), duration, temperature and source of DNA is expected to throw more light.

Investigation of the active oxygen species. Copper complexes can cleave DNA both through hydrolytic and oxidative processes. In the latter instance, these complexes have been shown to react with molecular oxygen or hydrogen peroxide to produce a variety of active oxidative intermediates (reactive oxygen species or ROS), including diffusible hydroxyl radicals and non-diffusible copper-oxene species (Surendra Babu *et al.*, 2007), while in others Fenton-type chemistry which involves release of diffusiable hydroxyl radicals has been suggested (Merfey *et al.*, 1981). To understand the active oxygen species in the reactions, degradation experiments with Rhodamine B dye (Fig. 4) was carried out. The degradation of the dye provides a direct measure of the concentrations of hydroxyl radicals in the reaction mixture. From the observation, it is suggested that reactive oxygen species can be produced by the copper complex in the presence of oxidant (Jie Liua *et al.*, 2002).

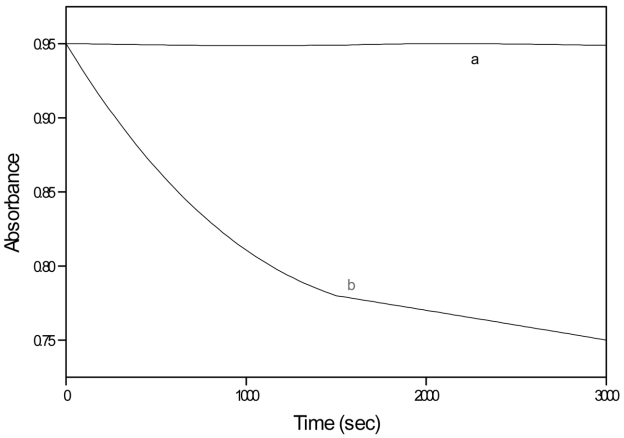

Fig. 4. Rhodamine B degradation followed by decrease of the absorbance at 552 nm at pH 8.1 in 10 mM phosphate buffer: (a) in presence of 0.1 mM $[CuL^1](OAc)_2$; (b) in presence of 0.1 mM $[CuL^1](OAc)_2$, 1 mM H_2O_2, 10 mM ascorbic acid.

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